Remarks

Claims 1-6 were pending and under consideration. With this Amendment, claims 1-6 are being amended. Upon entry of this Amendment, claims 1-6 will be pending. The amendments of the claims and the various rejections raised in the Office Action are discussed in more detail, below.

I. Amendments

A. In the Specification

The first sentence of the Abstract has been amended to replace "means of" with "using" to produce a grammatically complete sentence. The same amendment was made in the second sentence of the Abstract for purposes of continuity.

B. In the Claims

Claim 1 is amended to replace recitation of "1-3 days" with "1 day." Support for this amendment can be found, for example, on page 18, line 15 and on page 19, lines 15-16 of the originally filed specification.

Claims 2-6 were amended to replace "A" with "The" as the proper article as suggested by the Examiner

No new matter is added by way of these amendments.

II. Objections to the Claims

The Examiner objected to the use of a comma after "Agrobacterium" in line three of claim 1. The comma has been deleted.

The Examiner objected to the use of "A" at the beginning of claims 2-6. "A" has been replaced with "The" in each of claims 1-6.

In light of the amendments to the claims, Applicants respectfully request withdrawal of the claim objections.

III. Rejections under 35 U.S.C. §103

Claims 1-6 were rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Tanaka *et al.* (JP2001029075, hereinafter "Tanaka").

This rejection is respectfully traversed.

A. The Present Claims

The present claims relate to a method for transforming a monocotyledon, comprising the step of infecting a seed of the monocotyledon with an Agrobacterium which contains a desired recombinant gene, wherein the seed is a germinated seed. The seed is germinated by being subjected to pre-culture with a medium containing a plant growth factor for 1 day after sowing.

B. The Cited Art

Tanaka describe a method for transforming a rice seed comprising incubating the rice seed in a medium containing 2,4-D (a plant growth factor) for 5 days, followed by co-cultivation with Agrobacterium. Tanaka also describes transformation with Agrobacterium containing a desired recombination gene.

C. Analysis

C1. Legal Standard for Determining Obviousness Under 35 U.S.C. § 103(a)

Determining obviousness under 35 U.S.C. § 103(a) requires an objective analysis involving four factual inquiries, which include:

(a) determining the scope and content of the prior art,

(b) ascertaining the differences between the prior art and the claims in issue;

(c) resolving the level of ordinary skill in the art; and

(d) evaluating evidence of secondary considerations.

See Graham v. John Deere, 383 US 17, 18, 148 USPQ 459, 467 (1966); see also M.P.E.P. § 2141.

Further, the Office examination guidelines following the Court decision in KSR International Co. v. Teleflex Inc., 82 USPQ2d 1385, 1385 (US 2007) indicate that an issue to consider in assessing obviousness is whether a combination of prior art elements yields "predictable results." See Federal Register, Vol. 72, No. 195, October 10, 2007.

The issue in the instant analysis is whether one skilled in the art, having the asserted reference in hand, along with knowledge commonly available to skilled artisans, would arrive at Applicants' claims, in the absence of the teachings afforded by the instant specification. Applicants respectfully submit that the answer to this question is "no," for at least the following reasons.

The transformation of monocots using Agrobacterium has conventionally been recognized as difficult. At a minimum, this method required the used of dedifferentiated tissue. Additionally, prior to infection with Agrobacterium, it generally took 3 to 4 weeks to induce the necessary level of dedifferentiation. The presently claimed invention is based on the surprising result that efficient transformation was obtained when undamaged seeds were pre-cultured in 2,4-D for only one day. The unpredictable nature of this discovery led to its acceptance for publication in The Plant Journal (47:969-976 (2006), attached herein as Appendix A).

Tanaka teaches a method of pre-culturing rice seeds in a medium containing 2,4-D for 5 days. As shown in Figure 1a of Appendix A, significant formation of calli-like tissue of the scutellata has occurred by days 4 and 5. In contrast, after 1 day of pre-culturing in 2,4-D, the morphology of the seed has hardly changed. One of skill in the art would not have expected that a single day of pre-culturing would have adequately prepared the seed for transformation by Agrobacterium.

In addition, as a result of the ability to transform seeds after only 1 day of pre-culture, it was observed that there is more efficient selection of transgenic cells from the surrounding non-transformed cells. Untransformed cells that are sensitive to hygromycin will also grow when they are near the Agrobacterium transformed cells which are resistant to hygromycin. This will lead to the regeneration of a chimeric plant. However, with the unexpected ability to transform seeds after only one day of pre-culture comes the added advantage of obtaining a higher ratio of transformed cells to untransformed cells. In comparing Figure 2 of the instant application (1 day of pre-culturing), Figure 3 (2 days of pre-culturing), and Figure 4 (3 days of pre-culturing), one can see that the shorter the pre-culturing period, the higher ratio of transformed cells to untransformed cells (which do not express GFP). As a result, when a longer period of

pre-culturing is used, a smaller ratio of cells are transformed, leading to the production of chimeric plants with fewer transformed cells.

II. Conclusion

In view of the foregoing, claims 1-6 in the application are non-obvious over the cited art, are believed to satisfy all the criteria for patentability and are in condition for allowance. A Notice of Allowance is therefore respectfully requested.

No fees are believed due with this communication. However, the Commissioner is hereby authorized and requested to charge any deficiency in fees herein to Deposit Account No.: 110980.

If the Examiner has any questions or believes a telephone conference would expedite prosecution of this application, the Examiner is encouraged to call the undersigned at

Respectfully submitted,

Date: 6/25/08

/Susan L. Harlocker/

Registration No. 59,144

Correspondence Address:
Customer No. 79975

Exhibit A

Reference Cited in Substitute PTO form 1449
Serial No.: 10/594,130
Attorney Docket No.: 59150-8038

The Plant Journal (2006) 47, 969–976 doi: 10.1111/j.1365-313X.2006.02836.x

TECHNICAL ADVANCE

Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice

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Summary

Several approaches have recently been adopted to improve *Agrobacterium*-mediated transformation of rice, both to generate the large number of T-DNA insertion plants needed for functional analysis of the rice genome, and for production of rice with additional agronomical value. However, about 3 months of *in vitro* culture is still required for isolation of transgenic rice plants. Here, we report the competency of scutellum tissue from 1-day pre-cultured seeds for *Agrobacterium*-mediated transformation. Furthermore, early infection of rice seeds with *Agrobacterium* enhanced efficient selection of transformed calli. Using our system, we successfully regenerated transgenic rice plantlets within a month of the start of the aseptic culture of mature seeds. Our new system should reduce the somaclonal variation accompanying prolonged culture of rice cells in the dedifferentiated state and facilitate the molecular breeding of rice.

Keywords: ***Agrobacterium*, callus, rice, somaclonal variation, transformation.**

Introduction

The first reliable *Agrobacterium*-mediated transformation system for rice was reported in 1994 (Hiei *et al.*, 1994). Although that report was reproducible and was considered a breakthrough in the molecular biology of rice (reviewed by Hiei *et al.*, 1997; Komari *et al.*, 1998), the production of transgenic rice plants took 3 to 4 months from the beginning of tissue culture.

We have previously reported an improved culture medium and conditions for efficient *Agrobacterium*-mediated transformation of rice (Toki, 1997). Although we have used this protocol for the successful production of transgenic rice plants in both basic and applied research (Anai *et al.*, 2003; Ashikari *et al.*, 1999; Goto *et al.*, 1999; Ku *et al.*, 1999; Takahashi *et al.*, 2001; Yamanouchi *et al.*, 2002), our procedure relies on callus culture for the infection of *Agrobacterium* and subsequent selection of transformed cells. Similarly, despite several strategies to improve the transformation processes, isolation of transgenic rice plants currently requires about 3 months (Jeon *et al.*, 2000; Lee *et al.*, 1999; Sallaud *et al.*, 2003, 2004; Uze *et al.*, 1997).

The occurrence of genomic changes is associated with regeneration of plants from cells cultured *in vitro* in a dedifferentiated state for prolonged periods of time (Labra *et al.*, 2001). These undesired genomic changes (somaclonal variation) present obstacles to the functional analysis of the rice genome via T-DNA tagging as well as to the development of desired rice traits via transformation. Therefore, there is a need to shorten the period of tissue culture as far as possible to minimize somaclonal variation.

It has been reported that 2- to 3-week-old rice callus derived from scutellum tissue of mature seeds is competent for *Agrobacterium*-mediated transformation (Hiei *et al.*,

1994, 1997). In the present study, we analyzed the competency for *Agrobacterium* infection of 1- to 5-day-old rice seeds pre-cultured in media containing 2 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), and found that scutellum tissue from 1-day pre-cultured seeds is competent for *Agrobacterium*-mediated transformation. Furthermore, early infection of rice seeds with *Agrobacterium* enhanced the efficient selection of transformed calli, resulting in the establishment of a high-speed transformation system in rice.

Results

We first compared the germination process of rice seeds on media with or without 2,4-D (Figure 1a). Seeds started germinating 1 day (24 h) after inoculation (1D); at this stage 2,4-D had no significant effect on the germination process. Two days after inoculation (2D), small dedifferentiated tissues were detected at the scutellum tissue of seeds grown on a medium containing 2,4-D. On day 3 (3D), distinct de-regenerated cells (calli) could be detected and these calli

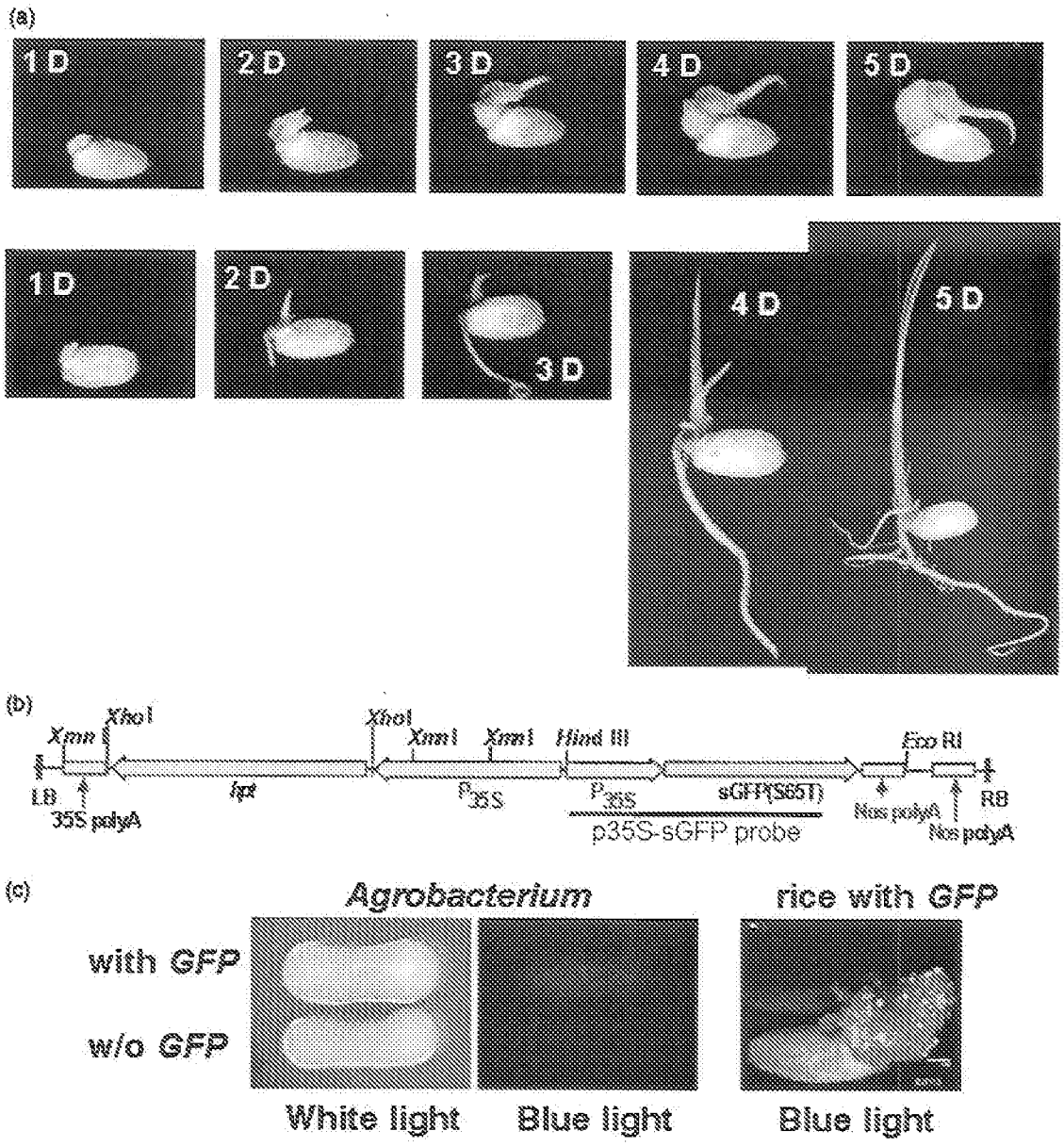

Figure 1. Pre-cultured plants, binary vector and *Agrobacterium* used in this study.
(a) The effect of 2,4-D in the culture medium on the germination process of mature rice seeds. Upper panels: with 2 mg l^{-1} 2,4-D; lower panels: without 2,4-D.
(b) The binary vector pCAMBIA1390-sGFP used in this study.
(c) Expression of the *gfp* reporter gene in *Agrobacterium* cells and plant cells. Although faint GFP signals were detected in *Agrobacterium* harboring pCAMBIA1390-sGFP, a much stronger signal was observed in transgenic rice calli.

developed further on days 4 and 5 (4D, 5D). We used these day 1 to day 5 seedlings for *Agrobacterium* infection.

In this experiment, we used the binary vector pCAMBIA1390-sGFP (Figure 1b), which contains a hygromycin resistance gene and a modified sGFP gene (Niwa, 2003; Niwa *et al.*, 1999) for selection of hygromycin-resistant calli and monitoring the fate of transformed cells, respectively. This construct was introduced into *Agrobacterium* EHA105 (Hood *et al.*, 1993), which was then used for the transformation study. Since only a faint GFP signal was detected from *Agrobacterium* EHA105 harboring pCAMBIA1390-sGFP, the strong GFP signals detected after infection of seedlings with *Agrobacterium* were considered to be derived from the plant cells rather than from the bacteria (Figure 1c).

Rice seeds pre-cultured for 1–5 days were inoculated with *Agrobacterium*, co-cultured for 3 days and then transferred to a selection medium containing carbenicillin to kill the bacteria and hygromycin to select for transformed plants. Growth of calli on the selection medium and emergence of GFP signals in seeds were observed on the 6th and 13th days following transfer to the selection medium (Table 1). Photographs were taken 7 and 14 days after transfer to the selection medium (Figure 2). Comparing the results of 6- and 13-day cultures showed similar patterns of callus growth and frequency of GFP expression on hygromycin-selective medium, indicating that the callus growth and GFP signals detected on the 6th day were derived from stable transformation of the transgene and not just from transient expression. Surprisingly, 1-day pre-cultured seedlings were already competent for *Agrobacterium*-mediated transformation (Table 1, Figure 2), although the transformation efficiency, as estimated from callus growth and the appearance of GFP signals, improved with the duration of the pre-culture period (Table 1). Seeds pre-cultured for 5 days were found to be most suitable for routine rice transformation.

Interestingly, early infection of rice seeds with *Agrobacterium* also enhanced the efficient selection of transformed calli. Indeed, vigorously dividing transformed cells were observed 14 days after transfer to the selection medium. These whitish-cream-colored embryogenic calli were compact but friable and thus ready for transfer to regeneration medium (Figure 2a,b). This was most probably due to the finding that early infection of rice seeds with *Agrobacterium* leads to early selection of transgenic cells, and this enables efficient selection of transgenic cells from the surrounding non-transformed cells.

Portions of the hygromycin-resistant GFP-expressing calli (about 25 mg of calli consisting of 10–20 pieces of small callus) were transferred to regeneration medium at 14 days after transfer to selection medium. Plantlets were regenerated for 10–14 days after transfer to regeneration medium (Figure 2a,b). When 1-day pre-cultured seeds were used for transformation, we could regenerate transformants within 30 days of the beginning of the culture. Regenerated plantlets were transferred to soil at 45 days from the beginning of culture and further grown to maturity. The growth and fertility of these plants in the greenhouse was comparable to that of non-transformed control plants, indicating the suppression of somaclonal variation in our system. The presence of the T-DNA in the T_0 generation of transgenic rice was confirmed by Southern blot analysis (Figure 3). The band sizes were different, indicating integration of the T-DNA at different locations in the rice genome. The copy number of integrated genes varied from one to three.

The stable inheritance of transgenes to the T_1 generation of transformed plants was evaluated by resistance to hygromycin and GFP expression. As shown in Figure 4 and Table 2, segregation ratios of 3:1 or 15:1 were observed for independent transgenic lines when resistant and sensitive seedlings were grown on an agar medium containing

Table 1 Effect of length of subculture on *Agrobacterium*-mediated transformation in rice

Subculture (days)	Number of co-cultured seeds	% co-cultured seeds yielding Hm-resistant calli (days after disinfection)		% GFP-expressing co-cultured seeds (days after disinfection)	
		(6 days)	(13 days)	(6 days)	(13 days)
1	119	17.6	20.2	12.6	15.1
2	105	31.4	38.1	23.8	26.7
3	114	60.5	57.0	64.0	64.9
4	107	72.9	74.8	77.6	76.6
5	110	98.2	98.2	85.5	95.5

Seeds were subcultured for 1–5 days and then co-cultured with *Agrobacterium* for 3 days. After disinfection, seeds were transferred to selection medium containing 50 mg l^{-1} hygromycin (Hm). At 6 and 13 days after transfer to selection medium, we counted the number of seeds inducing Hm-resistant calli and the number of seeds expressing GFP signal. Some seeds induced Hm-resistant calli without the GFP signal, while some seeds expressed GFP signal in scutellum without inducing visible calli.

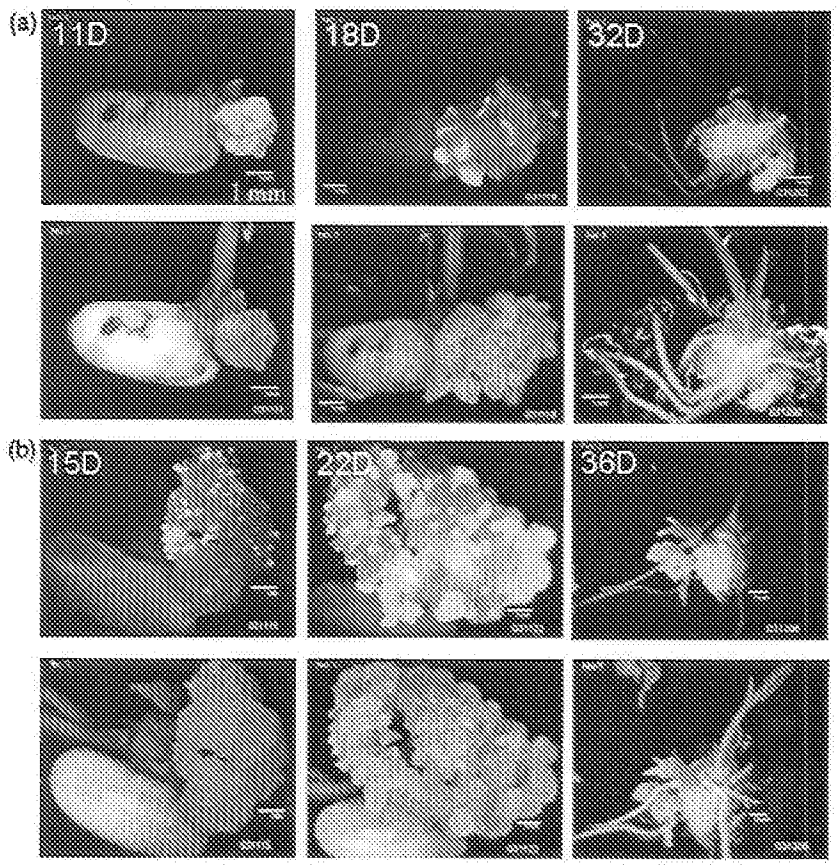

Figure 2. Production of transgenic calli after *Agrobacterium*-mediated transformation on 1- and 5-day pre-cultured rice seeds.
(a) Seeds were pre-cultured for 1 day (24 h), inoculated with *Agrobacterium*, co-cultured for 3 days and then subjected to hygromycin selection. Eleven days (11D) after sowing (7 days after co-culture), vigorously growing calli with strong GFP signals were observed. At 18 days (18D) after sowing, transgenic calli were transferred to regeneration medium; 32 days (32D) after sowing, regenerated plantlets from transformed callus were observed.
(b) Seeds were pre-cultured for 5 days, co-cultured with *Agrobacterium* for 3 days and then transferred to selection medium. At 15 days (15D) after sowing, vigorously growing calli with GFP signals were observed. At 22 days (22D) after sowing, transgenic callus was transferred to regeneration medium; 36 days (36D) after sowing, regenerated plantlets from transformed callus were observed. Upper panels: blue light; lower panels: white light.

hygromycin. Similarly, GFP-positive and GFP-negative progeny plants segregated according to a 3:1 or 15:1 ratio.

It has been reported that activity of retrotransposon Tos17 is associated with tissue culture-induced mutations in rice (Hirochika *et al.*, 1996). We assessed the copy number of Tos17 by Southern blot analysis (Figure S1). Our results indicate that the Tos17 copy number (there are two in Nipponbare) remained unchanged in transgenic plants produced using our protocol.

We next determined the exact time and location of transient or stable expression of the transgene. For this purpose, GFP signals in cultured rice seeds were examined daily following *Agrobacterium* infection. We used about 100 rice seeds and followed the expression of GFP signals in each seed. In this experiment, 1-day pre-cultured seeds were inoculated with *Agrobacterium* for 3 days and then transferred to selection medium. As shown in Figure 5, after 3 days of co-culture (Day 4), a faint but broad GFP signal was observed on the scutellum-derived callus. The GFP signal increased in intensity on the next day (day 5) but decreased on day 6. However, on day 7, a small but distinct and separate signal was again observed; this signal developed further day by day and never disappeared. Therefore, we speculate that the GFP signal observed on days 4 and 5 was derived from transient expression of the foreign gene (T-DNA) that was not integrated into the rice genome, and that these signals from non-integrated T-DNA then disappeared, probably due to digestion of non-integrated T-DNA on day 6. In contrast, the cells that had been stably transformed with T-DNA started growth via cell division and cell expansion on day 7 and GFP signals also then expanded day by day.

Discussion

Previously, it was believed that vigorously dividing calli that had been induced from rice scutellum and cultured for

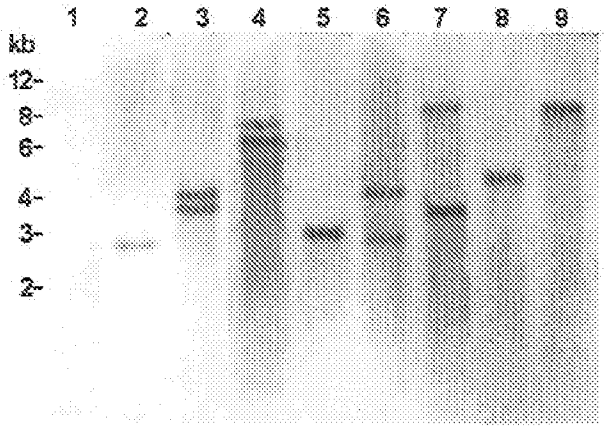

Figure 3. Southern blot analysis of transformants.
Genomic DNAs from a non-transgenic plant (lane 1) and T_0 generation transformants (lanes 2–9) were digested with *Xho*I and hybridized with the p35S-sGFP probe shown in Figure 1b. Lanes 2–9: DNAs from 1D-1, 2D-1, 3D-1, 3D-2, 4D-1, 4D-2, 5D-1 and 5D-2 plants. Abbreviations 1D-1, 2D-1 etc. are defined in Table 2.

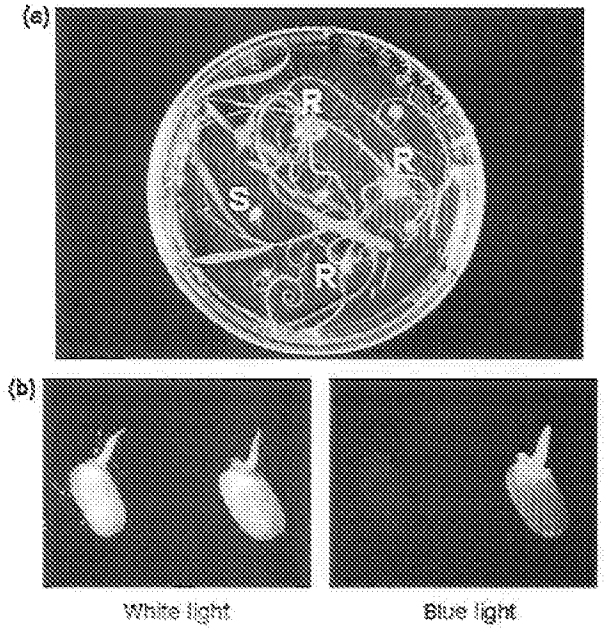

Figure 4. Segregation of hygromycin-resistant and -sensitive plants (a) or GFP-positive and -negative plants (b) in the T_1 generation of transgenic plants.

14–21 days on 2,4-D-containing medium were critical for *Agrobacterium*-mediated transformation. However, in the present study, we demonstrated that 1-day-old (day 1) rice seeds grown on a medium containing 2 mg l^{-1} 2,4-D were competent for *Agrobacterium*-mediated transformation. Furthermore, a very faint GFP signal on day 2 seeds was also detectable (data not shown).

Several factors could explain our success in transforming rice scutellum-derived tissue at a very early stage of pre-culture with extremely high frequency. We initially treated rice seeds with a relatively high concentration of sodium hypochlorite and Tween 20 detergent for disinfection; this process was repeated twice, although Tween 20 was not added in the second disinfection. We speculate that this disinfection step is not only effective in sterilizing the seeds but also removes various oils and waxes that might otherwise prevent the penetration of the scutellum by the bacteria.

Subsequently, we added a substantial amount of proline to the culture medium and maintained the temperature at 32–33°C throughout the culture except for the co-cultivation and regeneration steps. These culture conditions allow the rapid growth of cultured rice cells (Toki, 1997). Interestingly, proline is considered to provide protection against stress via various mechanisms (Hare and Cress, 1997; Nanjo *et al.*, 1999). Hence, proline might assist the recovery of rice cell growth after co-culture with *Agrobacterium*. Furthermore, it is also possible that the use of high temperatures stimulates the removal of bacteria from rice cells after co-culture, since plants become more resistant to infection by *Agrobacterium tumefaciens* at temperatures above 32°C (Braun, 1947; Fullner *et al.*, 1996).

Genomic changes in transgenic rice plants produced by conventional *Agrobacterium*-mediated transformation systems have been reported (Labra *et al.*, 2001). Furthermore, genotoxicity of 2,4-D has also been reported (Filkowski *et al.*, 2003), although this synthetic auxin is essential for the growth of cultured rice cells. However, a recent publication indicated that genomic modification of transgenic Arabidopsis plants by the floral dip method was comparable to that of non-transgenic plants (Labra *et al.*, 2004). In this context, our results indicate that the Tos17 retrotransposon insertion number, which has been reported to increase during prolonged periods of tissue culture (Hirochika *et al.*, 1996), was not changed in transgenic plants produced by our protocol. Furthermore, the growth and fertility of these plants in the greenhouse was comparable to that of non-transformed control plants, indicating that the somaclonal variation that usually accompanies tissue culture is suppressed in our system.

Although *in planta* transformation of rice using shoot apices has been reported (Park *et al.*, 1996), to date there have been no other confirmatory reports. The improved rice transformation system presented here is simple and reproducible. We have already produced more than 10 000 transgenic rice plants using our new method. Furthermore, our protocol works with several japonica varieties including Koshihikari, Dontokoi, and one indica variety, Basmati 370 (data not shown). Recently, reverse genetic approaches using T-DNA and the *Ds* transposon have been extensively used in investigations into the functional genomics of rice (An *et al.*, 2005; van Enckevort *et al.*,

Table 2 Estimation of T-DNA copy numbers in the transformants, and segregation pattern for hygromycin resistance and GFP expression in the progeny

Transformants[a]	Copy number in T_0 generation *gfp*[b]	GFP expression: Number of plants in T_1 generation +	GFP expression: Number of plants in T_1 generation −	GFP expression: Number of loci[c]	GFP expression: χ^2	Hygromycin resistance: Number of plants in T_1 generation R	Hygromycin resistance: Number of plants in T_1 generation S	Hygromycin resistance: Number of loci	Hygromycin resistance: χ^2
1D-1	1	31	13	1	0.273	31	13	1	0.273
1D-2	NT	51	16	1	0.005	51	16	1	0.005
2D-1	2	73	5	2	0.031	71	7	2	0.578
3D-1	2 or 3	54	3	2	0.001	54	3	2	0.001
3D-2	1	14	4	1	0.000	14	4	1	0.000
4D-3	NT	73	5	2	0.031	73	5	2	0.031
5D-2	1	55	10	1	2.713	55	10	1	2.713
5D-3	NT	71	24	1	0.004	71	24	1	0.004

[a]xD-y: xD means that the plants were derived from x-day-old seeds infected with *Agrobacterium*; y means they were plants derived from different seeds and therefore they were considered as independent transgenic plants.
[b]The copy number in the T_0 generation was determined by Southern blot analysis with a *gfp* probe.
[c]The number of loci was determined by GFP expression or hygromycin-resistant ratios that give the smallest χ^2 values.
R, resistant; S, susceptible; NT, not tested.

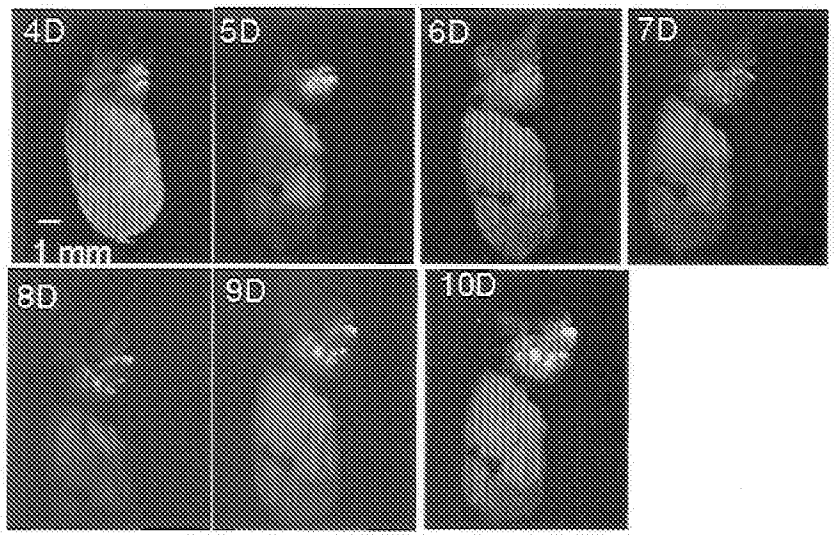

Figure 5. Transient and stable expression of the *gfp* gene after infection of *Agrobacterium* on 1-day pre-cultured rice seeds.
One-day pre-cultured rice seeds were inoculated with *Agrobacterium* and co-cultured for 3 days. GFP signals from 100 infected rice seeds were observed daily under a dissecting microscope. 4D indicates 4 days after sowing (3 days after inoculation of *Agrobacterium*). 5D indicates 5 days after sowing (1 day after disinfection of *Agrobacterium* by carbenicillin). 6D, 7D, 8D, 9D and 10D indicate 6, 7, 8, 9 and 10 days after sowing, respectively.

2005). Therefore, the use of our system should facilitate both studies on the functional genomics of rice and the development of transgenic rice with improved agronomic values.

Experimental procedures

Plasmid construct

The binary plasmid pCAMBIA1390-sGFP (Figure 1; A. Baba, National Institute of Agrobiological Sciences, Tsukuba, Japan and M. Ugaki, University of Tokyo, Japan, unpublished data) was constructed by inserting a 1.4 kb *HindIII/EcoRI* fragment containing the 35S-sGFP-nosT (green fluorescent protein coding sequence (Niwa, 2003; Niwa *et al.*, 1999) governed by the CaMV 35S promoter and nos terminator), into the *HindIII/EcoRI* site of the pCAMBIA1390 binary vector (Cambia, Canberra, Australia). The pCAMBIA1390-sGFP was transferred into *A. tumefaciens* strain EHA105 (Hood *et al.*, 1993) by electroporation.

Culture media

Supplementary Table S1 lists all the culture media used in the present study.

Plant materials and pre-culture

Mature seeds of the japonica rice cultivar 'Nipponbare' were used in this study. Dehulled seeds were sterilized with 70%

ethanol for 1 min prior to washing in sterile water. These seeds were further sterilized with 2.5% sodium hypochlorite containing 1 drop of Tween 20 per 50 ml for 15 min then washed five times in sterile water. This step was repeated once without Tween 20. The sterilized seeds were inoculated on N6D medium solidified with 0.4% Gelrite and cultured under continuous light at 32°C for 1–5 days.

Agrobacterium *infection and selection of transgenic plantlets*

Agrobacterium strain EHA105 harboring pCAMBIA1390-sGFP was cultured on AB medium containing 50 mg l^{-1} kanamycin sulfate solidified with 1.5% agar for 3 days at 28°C in the dark. One loop of *Agrobacterium* culture was scraped from the plates and suspended in AAM medium (Table 1) to yield an OD_{600} of approximately 0.1. Pre-cultured seeds were immersed in the *Agrobacterium* suspension by gently inverting the tube for 1.5 min, then blotted dry with a sterilized filter paper to remove excess bacteria. These seeds were transferred onto a sterilized filter paper (9-cm diameter) that had been moistened with 0.5 ml of AAM medium placed on 2N6-AS medium solidified with 0.4% Gelrite. After 3 days of co-cultivation at 25°C in the dark, seeds were washed five times in sterilized water and then washed once in sterilized water containing 500 mg l^{-1} carbenicillin (Wako Pure Chemicals, Osaka, Japan) to remove *Agrobacterium*. The seeds were rapidly blotted dry on a sterilized filter paper and cultured on N6D medium containing 50 mg l^{-1} hygromycin and 400 mg l^{-1} carbenicillin under continuous light at 32°C for 2 weeks. Proliferating calli arising from the scutellum were transferred to RE-III medium. Plantlets arising from the calli were transferred to HF medium to induce roots.

Southern blot analysis

Genomic DNA was extracted from 250 mg of fresh 2-month-old rice leaves using Nucleon PhytoPure plant and fungal DNA extraction kits (Amersham Life Science, Buckinghamshire, UK) according to the protocol provided by the manufacturer. Southern blotting analysis was performed according to standard protocols. DNA probes were prepared using a PCR digoxigenin probe synthesis Kit (Roche, Mannheim, Germany) according to the procedure recommended by the manufacturer.

Segregation analysis of transgenic plants

Selfed seeds (T_1 generation) of transformants were sown on agar medium, and GFP signals were observed under a fluorescence microscope. Germinated seeds were transferred to agar medium supplemented with 50 mg l^{-1} hygromycin sulfate to test hygromycin sensitivity.

Acknowledgements

We thank Y. Niwa for providing sGFP and A. Saba for providing pCAMBIA1390-sGFP. We thank H. Hirochika, M. Yamazaki and K. Sugimoto for providing the Tos17 clone. We thank H. Rothnie for language editing of this manuscript. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan. Part of this study was also supported financially by the Budget for Nuclear Research from the Ministry of Education, Culture, Sports, Science and Technology, based on screening and counseling by the Atomic Energy Commission.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. The copy number of Tos17 in transgenic plants is not increased during the short tissue culture period. Genomic DNA from a wild type plant and the T_0 generation of transformants were digested with *XhoI* and hybridized with a Tos17 probe. Lane 1: DNA from a wild-type plant. Lanes 2–9: DNAs from plants 1D-1,2D-1, 2D-2, 4D-1,4D- 2, 5D-1 and 5D-2. The abbreviations 1D-1, 2D-1 etc. are defined in Table 2. Two bands (10.4 and 15.3 kb) were detected in wild-type rice (Nipponbare) and the same number of bands was also detected in transgenic plants.

Table S1 All the culture media used in this study

This material is available as part of the online article from http://www.blackwell-synergy.com

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